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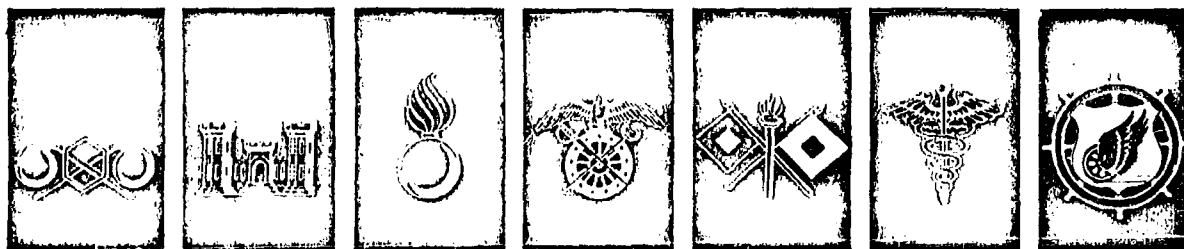
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TITLE: Wound Healing, Collagen, and Humoral Growth-Promoting Agents
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ABSTRACT: Physical, biochemical, surgical and histological techniques have been used by us in a series of studies on many aspects of wound healing. We have found that acetic acid solubilized collagen is poly-disperse and have fractionated this mixture into 3 high molecular weight components and a peptide complex. While the three high molecular weight components resemble the parent collagen molecule very closely in their amino acid composition, they do differ slightly but significantly from one another. The peptide fraction has been separated into some 14 fractions; they have little hydroxyproline and much proline, from which we postulate that they may represent an early stage in collagen develop-ment, that is, they are "precursors". Evidence to substantiate this hypothesis was obtained through metabolic studies using C^{14} glycine.

     We have isolated from dog wound fluid ultrafiltrate a low molecular weight component which is a growth accelerator for bacterial and mammalian cells.

     We have created an in-vivo collagen defect in rats by feeding them deuterium oxide. This technique has provided a tool for the study of the interaction of collagen with the ground substance; we are apply-ing this method to elucidate how the mucopolysaccharides of ground substance stabilize collagen, a vital subject about which little is now known.

WOUND HEALING, COLLAGEN, AND HUMORAL GROWTH PROMOTING AGENTS

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The healing of a wound is a dynamic process, involving mechanisms, both general and unique, which manifest themselves through a spectacular progression of biochemical and cellular changes during the healing period. One of the most striking of these phenomena is the creation of collagen, a substance absolutely vital to the healing process; this material has constituted our major field of interest in the past few years. We have attempted to approach the following questions: What is collagen? How is it synthesized and degraded by the living organism? What is the role of ascorbic acid in these processes, and what is the relationship of collagen to the "ground substance", that material in which the fibers lie buried, and which presumably confers on them dimensional stability?

Collagen is the fundamental structural material of the animal kingdom. One finds it listed in the biochemistry textbooks as a "scleroprotein", that is, one of those proteins including keratin which will dissolve in no known salt solution. It has been classically distinguished from the other members of its family by its unique ability to dissolve in hot water. It is now known that collagen will, to a certain extent, dissolve in salt solutions, and that this "salt soluble" collagen is itself heterogeneous. Thus, there exists a number of different collagen fractions derived from the parent protein macromolecule. This parent macromolecule is analagous to the more familiar protein-macromolecular fibers, such as silk or wool.

Collagen is apparently synthesized within the fibroblast and extruded into the extracellular spaces, where it develops by an as yet unknown process or processes into the giant, macromolecular, banded strands called collagen. Transverse striations are characteristic of collagen, and lie about 640 Å apart in native collagen. The amino acids hydroxyproline and hydroylysine constitute, apparently, another characteristic property unique to collagen. The collagen macromolecule has been considered to be a very long one composed of

the three inextricably intertwined strands, each with repeating sub-
units of the same structure. However, it has thus far been impossible
to isolate from the huge strands of the material a unit to which the
term "molecule" could be properly applied. The term "tropocollagen"
has been assigned to a theoretical building block which supposedly
exists in native collagen (1), and fragments of collagen have been
visualized by the ultramicroscope, which are thought to correspond to
"tropocollagen" (2). This "unit" however has never been isolated and
remains hypothetical.

It has been known since before 1870 (3) that collagen swells
in dilute acetic acid, loses its fibrillar appearance, and eventually
dissolves, in part, to yield a viscous solution. Within the last few
years American workers have postulated that dissolved collagen should
contain more than one species (4), and Russian investigators have
fractionated dissolved collagen into two components (5). Attempts
at detailed examination of the chemical and physical characteristics
of these components have been hindered by the inability to isolate
them adequately. The techniques (6) used to fractionate those sub-
stances known commercially as "gelatin"--collagens more or less dras-
tically treated with acids or alkalies--do not seem satisfactory for
this purpose. Ion exchange chromatography (7) requiring sodium
hydroxide is drastic for the study of primary structure; as for other
methods, Orekhovich and Shpikiter (8) found alcohol precipitation (9)
and coacervation (10) unsatisfactory for the separation of the compo-
nents demonstrated by ultracentrifugation of solutions of collagen
treated with hydrogen bond-breaking agents. We have developed a
method which we find satisfactory and have succeeded in chromato-
graphing rat tail tendon collagen into at least four major components.
The method involves gradient elution chromatography on warmed carboxy-
methyl cellulose columns, of acetic acid solubilized collagen, under
mild conditions of pH and low salt concentrations (11). Rat tail
tendons were cut into 1-cm pieces, directly immersed in 100 ml of
chilled 0.16 or 0.2 M sodium chloride and shaken slowly for 18 hours
at 4°. This sodium chloride extract was discarded, and the tendons
washed with 5 portions of chilled distilled water; each rinse was
50 ml and lasted 5 to 10 seconds. After the addition of 35 ml of 0.1 M
acetic acid to 240 mg of wet tendon, the mixture was allowed to stand
in a tightly stoppered Erlenmeyer flask at various temperatures and
for various times. The mixture was centrifuged and filtered. The
result was a clear viscous solution of collagen which was usually
chromatographed immediately, although no changes were detected in chro-
matographic patterns of solutions left 4 months at 4°C. The carboxy-
methyl cellulose was prepared from Whatman 200 mesh wood pulp cellu-
lose by the method of Peterson and Sober (12). Columns were 0.9 cm
in diameter and jacketed for water circulation allowing precise con-
trol of the 40° temperature required for the chromatography. After
the column had packed by gravity, it was washed with the starting
buffer until the effluent was no longer basic, and then with about
4 ml of distilled water, and allowed to become dry at the top.

A sample (1.5 ml) of the 0.1 M acetic acid collagen solution, containing about 0.5 mg of nitrogen, was applied to the heated column (40°) at this time. This was the first time that the collagen solution was subjected to a temperature higher than 20°-24°. The sample was allowed to run into the adsorbent, covered with 4 ml of initial buffer, and the elution gradient process was started. The increase of sodium ion in the elution gradient process was nearly linear for most of its course, starting at 0.05 M sodium ion, and reaching a maximum of 0.165 M sodium ion at the end of the chromatographic process. Two-milliliter fractions were collected at a flow rate of 28 ml per hour; each run took about 7 hours; recovery of collagen from the columns in one experiment in which all the fractions were combined, with Kjeldahl nitrogen as an index, was 104%. Amino acid analyses, with the exception of hydroxyproline, were performed by the chromatographic method of Moore and Stein (13) on columns of Amberlite IR-120, and determined quantitatively by the method of Rosen (14).

We found by this procedure that rat tail tendon collagen, dissolved in acetic acid actually consists of four major parts (Fig. 1). This acetic acid collagen solution, however, shows only one component in the ultracentrifuge. Three of the fractions we isolated are high molecular weight, gelatin-like components, each of which has the singular amino acid composition characteristic of the parent collagen, that is, high glycine, proline and hydroxyproline concentrations. The other fraction, however, is a mixture of small peptides, almost quantitatively dialyzable, containing much glycine and proline but little hydroxyproline, in distinct contrast to the other fractions.

In a number of runs, we carried out the entire procedure sterilely up to the chromatography itself; these chromatograms were identical to the ones in which no sterile precautions were taken. Bacterial processes, then, seem to play no role in the collagen fractionation achieved by our procedure.

The process by which the fractions are created seems to be an "all or none" one, since experiments showed that the characteristic four-fraction pattern appeared at the earliest time we could manage to complete the steps preliminary to chromatography (10 minutes). Such an "all or none" process was also suggested by Boedtker and Doty (15) for the breakdown of dissolved ichthyocol in warm citrate buffer. It seems reasonably certain that hydrolysis played no significant part in the production of the rat tail collagen components, since our chromatographic procedure required only 7 hours and the peptide fraction was completely eluted in as little as 10 minutes. The solutions retained their chromatographic properties after being kept at 25° C for 148 hours and changed only slightly when kept at 40° up to 48 hours. After 48 hours at 40° C the chromatograms were smeared; this may have been due to hydrolysis. Boedtker and Doty found what seems

to have been a similar process of hydrolysis for ichthyocol dissolved in pH 3.7 citrate buffer, the viscosity of which fell slowly with time when the solution was kept for over 40 hours at $36°$ C.

With respect to the chromatographic separation of the last three components, we have no evidence for the nature of the bonds cleaved, nor for the structural features upon which the fractionation depends. These features may be physical, for example, molecular weights, or chemical, for example, differences in frequency of occurrence of amide groups. Preliminary analysis indicates that the three last fractions differ slightly in amino acid composition. The unique amino acid composition of the peptide fraction has already been mentioned. Thus, none of these collagen components could correspond to "tropocollagen" or to any other single, repetitive collagen building block. Gallop, et al. (16) have recently suggested the existence of ester-like bonds within ichthyocol which give rise to nondialyzable components when split with hydroxylamine or hydrazine in such a way that peptide bonds are not disrupted. It is possible that such bonds are also involved in the phenomena which we are discussing.

The first fraction obtained from the relatively dilute acetic acid solutions is interesting in that it contains so few amino acids, and that its hydroxyproline content is so low. It might be imagined that this peptide fraction is a contaminant. This is unlikely, since (a) the tendons were washed prior to analysis with 0.2 M sodium chloride at $4°$ C for 24 hours, treatment which might be expected to dissolve any clinging contaminants and (b) the singular amino acid distribution of these peptides does not correspond to any known mammalian protein, including "classical" collagen. This component, then, is evidence of the existence of discrete atypical areas within the collagen molecule; its small hydroxyproline but great glycine and proline content gives us reason to postulate that it may represent an early stage of collagen formation and may, in fact, be the intermediate through which proline is converted to hydroxyproline. This postulate is based in part on the observations of Stetten (17), that the formation of the hydroxyproline of collagen is unusual since it is formed from proline at or near the site of its introduction into the macromolecule. Free exogenous hydroxyproline is not utilized in collagen formation.

To test this hypothesis and to determine the physiological significance of the other three collagen fractions as well, we performed tracer experiments with C^{14} glycine. Collagen was obtained from the tail tendons of young growing rats at various times (3 hours to 6 weeks) after they had been injected subcutaneously with the labeled amino acid. The collagen samples were solubilized in 0.1 M acetic acid. These solutions were chromatographed on carboxymethyl-cellulose at $40°$ C as already described. The four components were identified and measured in the eluate by the Lowry method and by liquid scintillation counting. Sequential analyses showed that while each of the four fractions attained its maximum radioactivity

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twelve hours after injection, two of the fractions attained striking-
ly higher specific activities than the others, and they lost these
activities more rapidly also (Fig. 2, 3). The two components show-
ing the unusually high turnover rates were the first fraction (pep-
tides) and the second fraction (first of the high-molecular weight
components).

Orekhovich, et al. reported recently an experiment in which
carboxyl-labeled glycine-C^{14} was injected into rats (5). Skin
collagen solubilized with citrate buffers was subsequently fractiona-
ted into two components ("α" and "β") by ammonium sulfate precipita-
tion of the partially urea-denatured solution of collagen. They found
that their α fraction had attained a specific activity about three
times that of the β component 12-15 hours after injection. It will be
recalled that our own two active components of rat tail tendon colla-
gen were twice as radioactive as our two less active ones 12 hours
after injection. It seems possible, therefore, that our second frac-
tion (first of the high molecular weight components) may correspond
to the α fraction of the Russian workers, and that their β fraction
is a composite of our fractions 3 and 4. Orekhovich and his colleagues
did not isolate a low molecular weight component.

It is clear, then, that not only is collagen inhomogeneous
physically, but that the fractionated components we have found were
formed and metabolized at different rates. The unusual amino acid
composition of the peptide fraction, together with its low molecular
weight and very high metabolic activity, supports our hypothesis that
it represents long sought for collagen precursor.

We have made a long series of studies of the peptide frac-
tion with highly automated equipment designed in this laboratory for
this particular function, but applicable to studies of amino acids
and peptides in general. The equipment makes use of ion exchange and a
gradient elution scheme devised by Piez and Morris (18) for use with
automatic flow and recording devices. We have simplified the proced-
ure by eliminating the use of highly unstable ninhydrin solutions, and
by eliminating troublesome mechanical deficiencies. Our procedure
uses a clog-free, stable ninhydrin solution prepared in air and light
and used with no protection necessary from these elements. This is an
adaptation of another method, also developed by us (14). Our experi-
ments have led us to the conclusion that there may be as many as
fourteen separate peptides within the small molecular weight fraction.
We are determining the amino acid composition of these peptides. This,
together with measurements of the specific metabolic activity of each,
should delineate more exactly the biological role of these peptides
and make it possible to reconstruct some aspects of the biosynthetic
processes which give rise to them.

It is our thought that ascorbic acid may exert its influence
on collagen formation at this point of the synthetic process. Although
it has been known for over 200 years that wounds do not heal in

patients with scurvy, the mechanism whereby vitamin C affects colla-
gen synthesis is unknown. Our working hypothesis is that ascorbic
acid influences collagen metabolism by regulating the formation of the
peptide precursors which we have mentioned, and the rate at which
their many proline residues are converted to hydroxyproline. If this
is so, it provides one of the few examples, or only example, of a
demonstrable peptide precursor to an eventual protein in mammals.
Thus, the "template" mechanism, currently postulated as the mechan-
ism of protein synthesis, might not be a universal one, after all,
since in its classical form it requires the synthesis of protein
whole, not in sections.

Why does a wound begin to heal, what sustains the healing
process, and what stops it? From time to time, evidence has been
presented by various investigators that humoral factors, perhaps ori-
ginating locally, are involved in these processes. But the evidence
has been inconclusive. During the past few years, working with
wound fluid provided to us by Dr. John S. Schilling of the Univer-
sity of Oklahoma Medical School, we have shown the existence of an
ultrafilterable growth accelerating substance, probably peptide,
during the rapidly "healing" phase after subcutaneous implantation
of cylinders of stainless steel wire mesh.

When these cylinders are implanted, they are quickly filled
with serum and fibrin and contain varying numbers of red blood cells,
macrophages, and leukocytes. There then evolves over the next several
weeks the full pattern of proliferating granulation tissue, complete
with fibroblasts, capillaries, and fiber formation. The picture
resembles closely that seen in healing wounds. The fluid which
bathes the interfaces of the proliferating fibro-collagenous tissues
may be obtained by aspiration from the interior of the cylinders. We
call this "wound fluid", and have obtained it at varying periods
during the time-course of fibro-collagenous incapsulation and inter-
nal obliteration of the space of the cylinder. As many as 20 cylin-
ders were implanted subcutaneously in dogs and from 0.5 to 2.0 ml of
wound fluid were collected from each cylinder. We were able to ob-
tain in this way large amounts of the fluid for a variety of analy-
tical procedures.

We found first that very small quantities of an ultrafil-
trate of the wound fluid obtained during the first week following
implantation of the wire-mesh cylinders accelerated dramatically the
growth of L. casei in a chemically-defined amino acid medium (Fig. 4).
Ultrafiltrates of plasma taken from the dogs at the same time the
wound fluids were obtained had no such effect. We then traced the
appearance, accumulation, and disappearance of this factor in the
wound fluid as healing progressed (Fig. 5). It is clear that the
growth-promoting effect is highest early and then declines progress-
ively though even at three weeks after implantation the effect is
significantly higher than that of plasma ultrafiltrates obtained at

this time.

We have partially isolated and partially characterized this growth accelerator. It is nonprotein, somewhat basic, water and n-butanol soluble, but insoluble in ethyl acetate and other organic solvents. It loses 50% of its activity after heating for 4 hours at 100° C. It is not a lipid, amino acid, simple sugar, glucosamine, kinetin, or a complex lipid. Our current postulate is that it is peptide in nature.

Our working thesis is that this substance speeds the transport of amino acids and other critical metabolites across the cell membrane and thereby speeds protein synthesis. We also feel that its significance goes beyond that of accelerating bacterial growth alone and that it may be fundamental for growth in general, wound healing and organ regeneration. We have adduced some confirmatory evidence for this hypothesis by our finding that this wound fluid ultrafiltrate accelerates the growth of mammalian (mouse fibroblast) as well as bacterial cells. Table I shows results of a typical study of this type, carried out for us by Dr. Andre D. Glinos of the Growth Physiology Laboratory of our Division. The "control" was a sample of plasma ultrafiltrate isolated from the dog when the wound fluid was taken. The wound fluid ultrafiltrate effected an almost two-fold increase in rate of cell division.

Finally, we have recently undertaken a study of the three-dimensional configuration of collagen, its in vivo stability and its relation to the ground substance, by the introduction of heavy water into the diet of animals. It is known that heavy water alters the stability of proteins in vitro. We became interested in heavy water as a tool for the study of collagen when we used it in an in vitro study of the viscosity characteristics of collagen. Rat tail tendon collagen was dissolved first in various concentrations of acetic acid in ordinary water. These solutions were then held at or near the "melt" temperature of collagen, about 39° C. This is the temperature at which such solutions very rapidly lose their viscosity, due to thermal rupture of the hydrogen bonds which hold the macromolecules in their typical elongated helical configuration. Breaking of these bonds results in the conversion of the collagen to a "random coil" shape, much more nearly spherical, and therefore of very low viscosity. We found, to our surprise, that when rat tail tendon collagen was dissolved in acidic heavy water, the "melt temperature" was raised by an appreciable amount. Solutions of collagen which would normally uncoil in an hour at 39° C would now take several days to undergo the same conversion. Heavy water, in essence, acted as a "helix" protector, probably by hydrogen bond reinforcement

We have now carried out experiments with heavy water in vivo where the stability of collagen depends not only on its own inherent characteristics, but also on its relationship to the relatively acidic

ground substance. Rats were subjected to our standard 5 cm dorsal skin incisions (19) plus the abdominal subcutaneous implantation of polyvinyl sponges (20) (Fig. 6a, 6b.) Granulation tissue consisting of newly formed capillaries, fibroblasts, ground substance and collagen fibers develops in the plastic sponges. The process is similar to that which occurs in the wire mesh cylinders and in the healing incisions. Excision of the sponge at any chosen time provides a specimen of pure reparative collagen.

The incisions and sponges were examined histologically using a variety of staining techniques. In addition, the tensile strengths of the incisions were measured by a technique devised in our laboratory and the sponges with their contained granulation tissue were subjected to various chemical analyses. Included among the latter analyses were measurements of hydroxyproline as an index of collagen. We found that although the histologic appearance and hydroxyproline content of the sponges of the deuterated animals were normal, the tensile strength of their wounds was about 40% lower than that of the controls (Table II). Surprisingly, when both sets of wounds were soaked in formaldehyde, the deuterated ones gained relatively more strength, indicating that the formaldehyde had corrected, probably by methylene bridges, a basic collagen structural deficiency in the wound of the deuterated animal. Histological examination of the deuterated wounds showed the collagen less organized and finer in structure than the normal. In addition, skin and tail tendons of these animals were more susceptible to attack by collagenase in vitro and were relatively less soluble in neutral salt solution. All these phenomena indicate that heavy water produced an in vivo abnormality of both reparative and developmental collagen, or of its interaction with the ground substance, or both.

In summary, we have applied to the problem of wound healing a wide variety of physical, biochemical, surgical, and histological techniques. We have succeeded in showing that dissolved collagen separates into discrete fractions, and that one of these is a peptide complex with an atypical amino acid content; we have separated these peptides into 14 fractions by ion exchange chromatography; we have shown that these fractions are metabolically distinct and postulate that they may represent stages in collagen development; it is possible that ascorbic acid, acting on the peptides, converts them from predominantly proline types to predominantly hydroxyproline types, creating hydrogen bonding sites by which the peptides are tied into the main part of the macromolecule. We have produced an apparently unique collagen defect by forcing mammalian collagen to develop in a milieu of heavy water. We hope, through further study of this defect, to discover basic facts concerning the tertiary structure of naturally occurring collagen, and how it interacts with the mucopolysaccharides of the ground substance. Finally, we have found, in mammalian wound fluid, a substance which accelerates bacterial growth and the division of mammalian fibroblasts, and which may act in vivo, as a "wound hormone."

Table I. Effect of Wound Fluid Ultrafiltrate on the Growth o f L
Strain Cells in the Absence of Serum Protein

Material Added to Basal Medium	Initial No. of Cells x 10^3	Final No. of Cells x 10^3	Growth Ratio = Final No. Cells / Initial No. Cells
None (Control)	300	448	1.5
Wound Fluid Ultrafiltrate 3%	300	717	2.4
Wound Fluid Ultrafiltrate 6%	300	805	2.7
Wound Fluid Ultrafiltrate 12%	300	805	2.7

Table II

Fresh Wounds		Formaldehyde Fixed	
D_2O	Control	D_2O	Control
3.50	5.60	6.80	7.20
4.00	6.60	10.60	8.00
3.55	4.85	8.65	12.00
3.65	5.95	5.85	11.75
4.65	3.95	7.00	11.00
5.15	4.50	6.05	11.05
3.00	5.25	9.10	7.95
3.75	5.80	8.50	8.00
3.50	4.50	9.05	5.10
4.45	5.85	8.00	7.80
4.70	5.90	5.20	6.05
4.90	6.55	6.85	6.30
3.40	4.10	7.50	6.05
4.00	5.20	6.00	5.40
3.25	7.65	6.75	7.40
3.50	8.00	8.30	7.50
3.95 (Ave.)	5.64 (Ave.)	7.49 (Ave.)	8.04 (Ave.)
S.D. = ± 0.65	S.D. = ± 1.16	S.D. = ± 1.46	S.D. = ±2.24
S.E. = ± 0.16	S.E. = ± 0.29	S.E. = ± 0.37	S.E. = ±0.56

$P < 0.001$ $P > 0.1$

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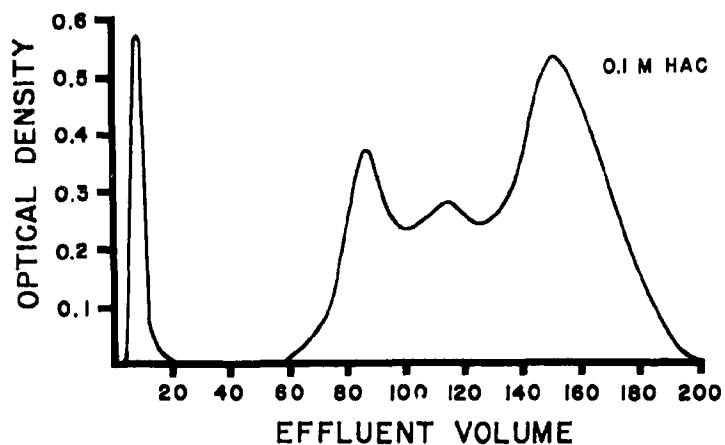20. Boucek, R. J., and Noble, N. L., A.M.A. Arch. Path., 59: 533,1955.

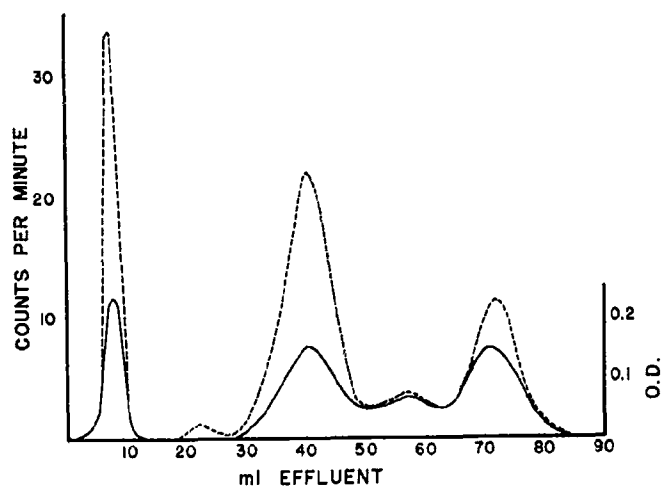Fig. 1 -- Chromatographic pattern of acetic acid solubilized rat tail collagen.

Fig. 2 -- Chromatographic and radioactivity patterns; rats injected with C^{14} glycine.
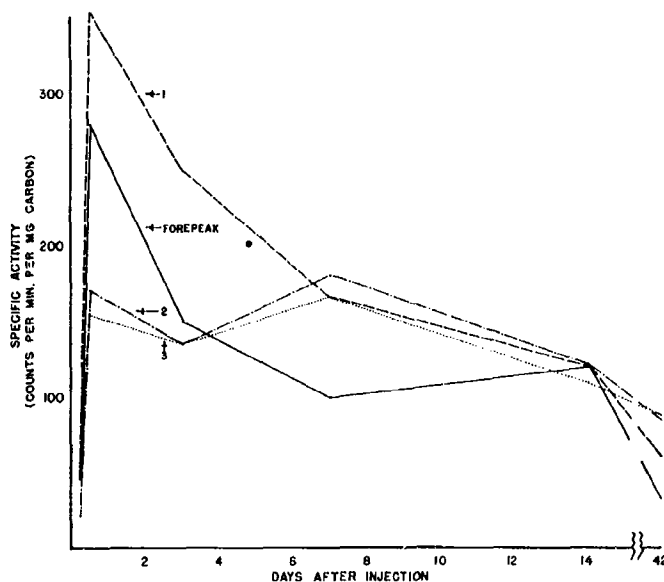
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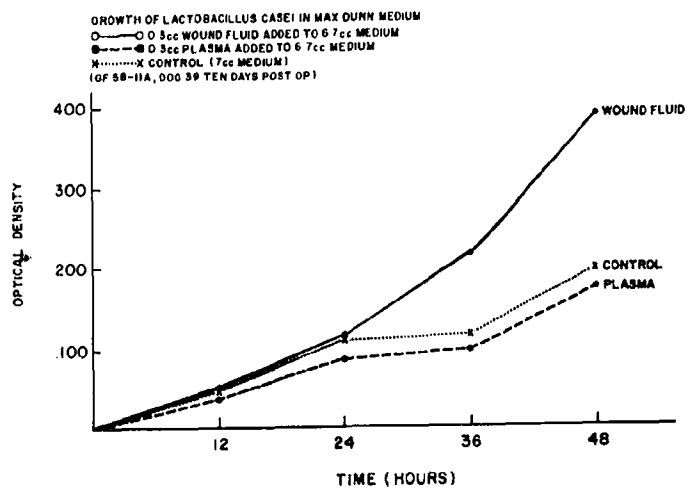Fig. 3 -- Rate of appearance and disappearance of C^{14} activity from fractions of tail collagen.

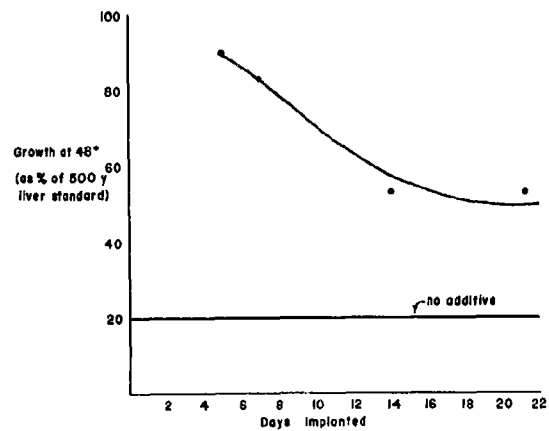Fig. 4 -- Acceleration of L. casei growth by dog wound fluid ultrafiltrate.

Fig. 5 -- Effect of implantation period on growth accelerating properties of wound fluid.
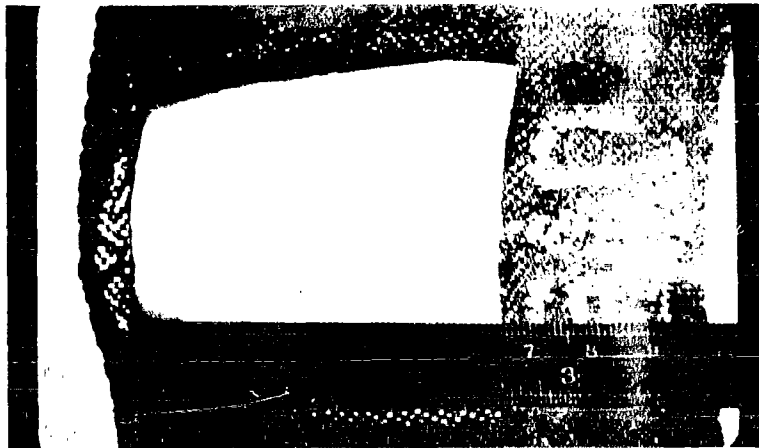

Fig. 6A -- Five cm. dorsal skin incision in the rat.

Fig. 6B -- Implantation of 25 mg polyvinyl sponge into the rat.